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SAM 317

Supplemental Assay Method for Titration of Canine Adenovirus in Canine Kidney Cell Culture

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Table of Contents

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1. Introduction

This Supplemental Assay Method (SAM) describes an ***in vitro*** test method for assaying modified-live canine adenovirus (CAV) vaccines for viral content. CAV endpoint is determined by viral cytopathic effect (CPE) in a Madin-Darby canine kidney (MDCK) cell line.

2. Materials

2.1 Equipment/instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

2.1.1 Incubator, 36° ± 2°C, high humidity, 5% ± 1% CO_2 (Model 3336, Forma Scientific Inc.)

2.1.2 Water bath, 36° ± 2°C

2.1.3 Microscope, inverted bright light (Model CK, Olympus America Inc.)

2.1.4 Vortex mixer (Vortex-2 Genie, Model G-560, Scientific Industries Inc.)

2.1.5 Syringe, self-refilling, repetitive, 2-ml

2.1.6 Pipettor with tips and/or motorized microliter pipette

2.1.7 Micropipettor, 300 µl x 12-channel

2.1.8 Pipette-aid

2.2 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below. All reagents and supplies must be sterile.

2.2.1 CAV Reference: Mirandola strain of CAV Type 1 or Manhattan strain of CAV Type 2 [available from the Center for Veterinary Biologics (CVB)]

2.2.2 Monospecific antisera, free of CAV antibody, that neutralize the non-CAV fractions present in multifraction vaccines, e.g. canine parainfluenza virus (CPI), canine parvovirus (CPV), canine distemper virus (CDV), etc. [available from CVB]

2.2.3 MDCK cell line, free of extraneous agents as tested by the Code of Federal Regulations, Title 9 (9 CFR)

2.2.4 Minimum essential medium (MEM)

2.2.4.1 9.61 g MEM with Earles salts without bicarbonate

2.2.4.2 1.1 g sodium bicarbonate ($NaHCO_3$)

2.2.4.3 Dissolve with 900 ml deionized water (DW).

2.2.4.4 Add 5.0 g lactalbumin hydrolysate or edamine to 10 ml DW, heat to 60°± 2°C until dissolved, and add to the solution in **Section 2.2.4.3** with constant mixing.

2.2.4.5 Q.S. to 1000 ml with DW and adjust pH to 6.8-6.9 with 2N hydrochloric acid (HCl).

2.2.4.6 Sterilize through a 0.22-µm filter.

2.2.4.7 Aseptically add 50 µg/ml gentamicin sulfate

2.2.4.8 Store at 2°- 7°C.

2.2.5 Growth Medium

2.2.5.1 940 ml MEM

2.2.5.2 Aseptically add:

1. 50 ml gamma-irradiated fetal bovine serum (FBS)

2. 10 ml L-glutamine (200 mM)

2.2.5.3 Store at 2°- 7°C.

2.2.6 Dulbecco's phosphate buffered saline (DPBS)

2.2.6.1 8.0 g sodium chloride (NaCl)

2.2.6.2 0.2 g potassium chloride (KCl)

2.2.6.3 0.2 g potassium phosphate, monobasic, anhydrous (KH_2PO_4)

2.2.6.4 0.1 g magnesium chloride, hexahydrate ($MgCl_2 \cdot 6H_2O$)

2.2.6.5 Dissolve reagents in **Section 2.2.6.1** through **Section 2.2.6.4** with 900 ml DW.

2.2.6.6 Add 1.03 g sodium phosphate, dibasic anhydrous (Na_2HPO_4) to 10 ml DW, heat to 60°± 2°C until dissolved. Add to **Section 2.2.6.5** with constant mixing.

2.2.6.7 Dissolve 0.1 g calcium chloride, anhydrous ($CaCl_2$) with 10 ml DW and add slowly to **Section 2.2.6.5** to avoid precipitation.

2.2.6.8 Q.S. to 1000 ml with DW; adjust pH to 7.0-7.3 with 2N HCl.

2.2.6.9 Sterilize through a 0.22-µm filter.

2.2.6.10 Store at 2°- 7°C.

2.2.7 Cell culture plates, 96-well

2.2.8 Polystyrene tubes, 12 x 75-mm

2.2.9 Pipettes, 10-ml

2.2.10 Reagent reservoir

2.2.11 Syringe, 1 ml tuberculin

2.2.12 Needles, 18-gauge x 1 1/2-inch

3. Preparation for the test

3.1 Personnel qualifications/training

Personnel shall have experience in the preparation and maintenance of cell culture, as well as in the propagation and maintenance of animal viruses and the quantitation of virus infectivity by CPE.

3.2 Preparation of equipment/instrumentation

On the day of test initiation, set the water bath at 36°± 2°C.

3.3 Preparation of reagents/control procedures

3.3.1 Preparation of MDCK cell culture plates (MDCK Plates)

Cells are prepared from healthy, confluent MDCK cells. On the day of test initiation, using a 12-channel micropipettor, add 200 µl/well of $10^{4.7}$ to $10^{5.2}$ cells/ml suspended in Growth Medium into all wells of a 96-well cell culture plate. Prepare 1 MDCK Plate for the controls and the first Test Vaccine. Each additional MDCK Plate allows testing of 3 additional Test Vaccines. Incubate at 36°± 2°C in a CO_2 incubator and use within 4 hours.

3.3.2 Preparation of CAV Reference Control

3.3.2.1 On the day of test initiation, rapidly thaw a vial of CAV Reference in the water bath.

3.3.2.2 Dispense 1.8 ml MEM into sufficient 12 x 75-mm polystyrene tubes to bracket the expected endpoint according to the Center for Veterinary Biologics (CVB) Reagent Data Sheet, and label (for example: 8 tubes labeled 10^{-1} through 10^{-8}, respectively).

3.3.2.3 With a 200-µl pipettor, transfer 200 µl of the CAV Reference to the first tube labeled 10^{-1}; mix by vortexing.

3.3.2.4 Using a new pipette tip, transfer 200 µl from the 10^{-1} labeled tube **(Section 3.3.2.3)** to the 10^{-2} tube; mix by vortexing.

3.3.2.5 Repeat **Section 3.3.2.4** for each of the subsequent dilutions, transferring 200 µl from the previous dilution to the next dilution tube until the tenfold dilution series is completed.

3.4 Preparation of the sample

3.4.1 The initial test of a Test Vaccine will be with a single vial (a single sample from 1 vial). On the day of inoculation, using a sterile 1.0-ml syringe and an 18-gauge x 1 1/2-inch needle, rehydrate a vial of the Test Vaccine with the provided diluent by transferring 1.0 ml for a 1-ml-dose vaccine, 0.5 ml for 1/2-ml-dose vaccines, etc., into the vial containing the lyophilized Test Vaccine; mix by vortexing. Incubate for 15 ± 5 minutes at room temperature.

3.4.2 For multifraction CAV vaccines, neutralize the non-CAV fractions with antiserum specific to each virus fraction.

3.4.2.1 Prepare a dilution of each neutralizing non-CAV antiserum in DPBS according to the CVB Reagent Data Sheet or as determined for that specific antiserum.

3.4.2.2 Dispense 200 µl of each of the required neutralizing antiserum into a 12 x 75-mm polystyrene tube labeled 10^{-1} and q.s. to 1.8 ml with MEM. For example, to neutralize 3 non-CAV viral components of a CDV/CAV/CPI/CPV vaccine, dispense 200 µl of each of the diluted CDV, CPI, and CPV antisera into the tube labeled 10^{-1}; add 1.2 ml of MEM to obtain a final volume of 1.8 ml.

3.4.2.3 Pipette 200 µl of the reconstituted Test Vaccine to the labeled tube to yield a 10^{-1} dilution; mix by vortexing.

3.4.2.4 Incubate at room temperature for 30 ± 5 minutes.

3.4.3 For vaccines containing only the CAV fraction, the 10^{-1} dilution is prepared by adding 200 µl of the Test Vaccine to 1.8 ml of MEM in a 12 x 75-mm polystyrene tube, labeled 10^{-1}; mix by vortexing.

3.4.4 Serial tenfold dilutions

3.4.4.1 Using a 2-ml self-refilling repetitive syringe, dispense 1.8 ml MEM into each of 4, 12 x 75-mm polystyrene tubes labeled 10^{-2} through 10^{-5} (or more if the expected CAV endpoint of the Test Vaccine is higher than 10^{-5}).

3.4.4.2 Using a new pipette tip, transfer 200 µl from the tube labeled 10^{-1} to the next dilution tube labeled 10^{-2}; mix by vortexing.

3.4.4.3 Repeat **Section 3.4.4.2** to the remaining tubes, transferring 200 µl from the previous dilution to the next dilution tube until the tenfold dilution series is completed.

4. Performance of the test

4.1 Label the MDCK Plates and inoculate each of 8 wells/dilution with 25 µl of the Test Vaccine, starting with the highest dilution (most dilute). In a similar manner, inoculate 8 wells/dilution of the CAV Reference Control (with dilutions 10^{-8} through 10^{-5} for the example in **Section 3.3.2.2**). Change tips between each unique sample

(i.e., each Test Vaccine and the CAV Reference Control), but tip changes are not necessary between each dilution in a series if pipetting from the most dilute to the most concentrated within that series (e.g., 10^{-8} through 10^{-5}). This becomes the Test Plate. Additional Test Vaccines may be inoculated onto other MDCK Plates in a similar manner, 3 Test Vaccines per Test Plate.

4.2 Eight uninoculated wells on the initial Test Plate serve as a Negative Cell Control.

4.3 Incubate the Test Plates in a 36°± 2°C CO_2 incubator for 11 days ± 1 day.

4.4 After incubation, read the Test Plate at 100X or 200X magnification on an inverted light microscope and examine cells for CPE. CAV CPE is characterized by cell rounding and lysis.

4.4.1 Wells displaying 1 or more areas of CPE are considered to be positive for CAV.

4.4.2 Record results as the number of CPE positive wells versus total number of wells examined for each dilution of the Test Vaccine and the CAV Reference Control.

4.5 Calculate the CAV endpoints of the Test Vaccine and the CAV Reference Control using the method of Spearman-Kärber as modified by Finney. The titers are expressed as $\log_{10}$, 50% tissue culture infective doses ($TCID_{50}$).

Example:

10^{-2} dilution of Test Vaccine = 8/8 wells CPE positive
10^{-3} dilution of Test Vaccine = 5/8 wells CPE positive
10^{-4} dilution of Test Vaccine = 1/8 wells CPE positive
10^{-5} dilution of Test Vaccine = 0/8 wells CPE positive

Supplemental Assay Method for Titration of Canine Adenovirus in Canine Kidney Cell Culture

Spearman-Kärber formula:

Test Vaccine Titer = (x – d/2 + [d • S]), where:

X = $\log_{10}$ of dilution with all wells CPE positive (2)
d = $\log_{10}$ of dilution factor (1)
S = sum of proportions of wells CPE positive for all dilutions tested:

$$\frac{8}{8} + \frac{5}{8} + \frac{1}{8} + \frac{0}{8} = \frac{14}{8} = 1.75$$

Test Vaccine titer = (2 – 1/2) + (1 • 1.75) = 3.25

Adjust the titer to the recommended Test Vaccine dose as follows:

A. divide the **Test Vaccine Dose** by the **Inoculation Dose**

Test Vaccine Dose = manufacturer's recommended vaccination dose (for this test CAV vaccine, the recommended dose is 1 ml)

Inoculation Dose = amount of diluted Test Vaccine added to each well of the Test Plate (for this test CAV vaccine, the inoculation dose is 0.025 ml)

$$\frac{1 \text{ ml dose}}{0.025 \text{ ml}} = 40$$

B. calculate $\log_{10}$ of value in **A** and add it to the **Test Vaccine titer** as illustrated below:

Log of 40 = 1.6

Test Vaccine Titer = 3.25 + 1.6 = 4.85

Therefore the titer of the **CAV Test Vaccine** is $10^{4.85}$ $TCID_{50}$/ml.

5. Interpretation of the test results

5.1 Valid Assay

5.1.1 The calculated titer of the CAV Reference Control must fall within plus or minus 2 standard deviations (± 2 SD) of its mean titer, as established from a minimum of 10 previously determined titers.

5.1.2 The lowest inoculated dilution of the CAV Reference Control must induce CPE in 100% of the wells (8/8). If an endpoint is not reached (1 or more wells are CPE positive at the highest dilution), the titer is expressed as "greater than or equal to" the calculated titer. If an endpoint is critical to testing, the highest (most dilute) must exhibit no CPE (0/8).

5.1.3 The Uninoculated Cell Control must not exhibit any CPE, degradation, or cloudy media that would indicate contamination.

5.2 If the validity requirements are not met, then the assay is considered a **NO TEST** and can be retested without prejudice.

5.3 In a valid test, if the titer of the Test Vaccine is greater than or equal to the titer contained in the Animal and Plant Health Inspection Service (APHIS) filed Outline of Production for the product under test, the Test Vaccine is considered **SATISFACTORY.**

5.4 In a valid test, if the titer of the Test Vaccine is less than the required minimum contained in an APHIS filed Outline of Production for the product under test, the Test Vaccine is retested in accordance with 9 CFR, Part 113.8.

6. Report of test results

Results are reported as $TCID_{50}$ per dose of Test Vaccine.

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.305, U.S. Government Printing Office, Washington, DC, 2004.

7.2 Cottral, GE, (Ed.). *Manual of standardized methods for veterinary microbiology*. Comstock Publishing Associates, Ithaca and London, 1978, pg. 731.

7.3 Finney, DJ, 1978. *Statistical method in biological assay.* Griffin, London. 3rd ed., 1978, pp. 394-401.

8. Summary of revisions

This document was revised to clarify the practices currently in use at the Center for Veterinary Biologics and to provide additional detail. While no significant changes were made that impact the outcome of the test, the following changes were made to the document:

- **2.2.4.2** The amount of sodium bicarbonate ($NaHCO_3$) has been changed from 2.2 g to 1.1 g.
- **2.2.4.7** Penicillin and streptomycin have been deleted.
- **4.5** Additional steps have been added to clarify the titer calculations by the Spearman-Kärber formula.
- **5.1.2** Recording the rate of positive reaction for validity requirements.
- The refrigeration temperatures have been changed from 4° ± 2°C to 2° – 7°C. This reflects the parameters established and monitored by the Rees system.
- "Test Serial" has been changed to "Test Vaccine" throughout the document.
- "Reagent and Reference Sheet" has been changed to "Reagent Data Sheet" throughout the document.
- The footnotes have been deleted with any pertinent references now noted next to the individual items.